

Copper induced oxidative stress in *Saccharomyces cerevisiae* targets enzymes of the glycolytic pathway

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Abstract Increased cellular levels of reactive oxygen species are known to arise during exposure of organisms to elevated metal concentrations, but the consequences for cells in the context of metal toxicity are poorly characterized. Using two-dimensional gel electrophoresis, combined with immunodetection of protein carbonyls, we report here that exposure of the yeast *Saccharomyces cerevisiae* to copper causes a marked increase in cellular protein carbonyl levels, indicative of oxidative protein damage. The response was time dependent, with total-protein oxidation peaking approximately 15 min after the onset of copper treatment. Moreover, this oxidative damage was not evenly distributed among the expressed proteins of the cell. Rather, in a similar manner to peroxide-induced oxidative stress, copper-dependent protein carbonylation appeared to target glycolytic pathway and related enzymes, as well as heat shock proteins. Oxidative targeting of these and other enzymes was isoform-specific and, in most cases, was also associated with a decline in the proteins' relative abundance. Our results are consistent with a model in which copper-induced oxidative stress disables the flow of carbon through the preferred glycolytic pathway, and promotes the production of glucose-equivalents within the pentose phosphate pathway. Such re-routing of the metabolic flux may serve as a rapid-response mechanism to help cells counter the damaging effects of copper-induced oxidative stress.

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Key words: Yeast; Oxidative damage; Protein oxidation; Copper toxicity; Proteome

1. Introduction

Copper is one of the essential trace elements, required as a cofactor for a number of enzymes and other cellular activities, including the uptake of iron [1–4]. In excess, however, copper is toxic and potentially carcinogenic. Neurodegenerative disorders, autism and Alzheimer's disease are some of the conditions that have been linked with copper exposure in humans [5–8]. It is widely considered that copper exerts its effects at the cellular level at least in part through induction of oxidative stress [9–12]. Reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxide anion (O₂^{•-}), and hy-

droxyl radical (OH[•]) arise normally during aerobic metabolism (with an estimated 2% of oxygen utilized by the yeast cell being converted into the superoxide anion [13]). However, ROS have the potential to cause oxidative damage to proteins, nucleic acids and other macromolecules, which can severely compromise cell health and viability [14]. Of necessity, therefore, aerobic organisms (eukaryotic and prokaryotic) have developed a network of defense mechanisms to protect against ROS. These include ROS-scavenging molecules (e.g. superoxide dismutases, catalases), oxidative damage-repair enzymes (e.g. methionine sulfoxide reductase) [9–12], and mechanisms such as the S-thiolation of oxidation-susceptible proteins, which prevents oxidation by forming reversible mixed-disulfide bonds with glutathione/thiols [15]. Environmental stresses such as irradiation and exposure to heavy metals are known to promote ROS formation in cells, potentially overwhelming antioxidant defenses. This may be particularly true of redox-active metals, like copper, which catalyze the Fenton reaction and can accelerate generation of the highly damaging OH[•] radical from O₂^{•-} and H₂O₂ substrates [14,16]. Oxidative damage to lipid membranes, for example, has previously been identified as one mode of copper action in the yeast model *Saccharomyces cerevisiae* [17,18]. Proteins are also major targets of ROS in cells, either through oxidation of their amino acid side chains to hydroxy or carbonyl derivatives, or by a shearing of their peptide bonds [19,20]. Moreover, individual proteins may display differing susceptibilities to oxidative attack, linked to variable compositions of sulfhydryl groups, Fe–S clusters, reduced heme moieties and Cu prosthetic groups [21]. Recently there has been a considerable effort to characterize the major cellular protein targets of ROS. A number of reports have attempted to address this issue in yeast, by determining some of the proteins that were either oxidatively damaged or specifically modified (S-thiolated) by treatment with H₂O₂ [19,20,22]. The assay for protein carbonyl content is particularly useful since this modification records relatively accurately on the fraction of oxidatively damaged protein with impaired function in total-protein samples [23]. In this report we ascertain, for the first time, which proteins are specifically carbonylated in the presence of subcritical, growth-inhibitory levels of copper. We found that there were marked temporal differences in the targeted oxidation of susceptible proteins during copper treatment, and that copper-dependent protein oxidation was isoform-specific. Moreover, the results indicate that a re-routing of metabolic flux likely occurs in cells exposed to non-lethal levels of copper, which may serve as a protective response to copper-dependent oxidative stress.

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2. Materials and methods

2.1. Strains, media and copper exposure

S. cerevisiae BY4741 (*MATa his3Δ1 leu2Δ;0 met15Δ0 ura3Δ0*) was routinely maintained on YEPD agar. Experimental cultures in YEPD broth were inoculated from 24 h starter cultures derived from single colonies and grown overnight to exponential phase ($OD_{600} \sim 2.0$) at 30°C with orbital shaking (120 rev min⁻¹) [11]. At the start of experiments, copper nitrate, Cu(NO₃)₂, was added to flasks to a final concentration of 8 mM and cultures were incubated with shaking as above. Growth was monitored with optical density at 600 nm. For determination of cell viability, aliquots of cell suspension were removed, diluted and plated on YEPD agar. Colony-forming ability was determined after 4 days at 30°C. At intervals during copper exposure, cell samples were harvested by centrifugation and flash-frozen.

2.2. Preparation of protein extracts

Frozen cell pellets were resuspended in lysis buffer (100 mM Tris, pH 7.4, 10% (v/v) glycerol, 1 mM PMSF, 264 mg ml⁻¹ aprotinin, 20 mg leupeptin, 10 mg ml⁻¹ pepstatin). Cells were disrupted with glass beads (0.5 mm diameter) using a mini-bead-beater (Biospec Products), interspersed with cooling on ice. Cell debris was removed by centrifugation (12000×g, 10 min, 4°C) and supernatants retained for protein analyses. Protein concentrations in the supernatants were determined according to Bradford [24].

2.3. Two-dimensional gel electrophoresis and Western blot analysis

The general methods for 2D protein analysis and Western blotting are adapted from Ausubel et al. [25] with modifications for immunodetection of protein carbonyl groups according to [20,26]. For 2D analysis either 40 μg (for protein detection) or 100 μg (for Western blotting) of protein was mixed with re-hydration solution (8 M urea, 0.4% dithiothreitol (DTT), 4% CHAPS and 1% immobilized pH gradient (IPG) buffer, pH 3–10) and loaded on to 18 cm IPG strips (non-linear pH gradient 3–10; Amersham Pharmacia). Isoelectric focusing was performed according to the protocol of Gorg et al. [27]. Strips containing samples intended for Western blotting were derivatized with 2,4-dinitrophenylhydrazine (DNPH) by equilibration in 10 ml of 10 mM DNPH in 0.2 N HCl for 20 min. All protein-laden strips were then equilibrated in sodium dodecyl sulfate (SDS) buffer (50 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 1% dithiothreitol for 15 min, and containing 4% iodoacetamide for an additional 15 min. Proteins within the strips were resolved further by electrophoresis through 12.5% homogenous polyacrylamide gels. For visualization of total proteins (40 μg protein samples), the 2D protein arrays were stained overnight with SYPRO Ruby[®] fluorescent protein-gel stain, as provided (Molecular Probes). Fluorescence from the proteins was imaged and quantified using the 2D Master Imaging System and Decyder analysis software 4.0 (Amersham Pharmacia). For analysis of carbonylated proteins by Western blotting, the derivatized proteins were electroblotted onto PVDF membranes. The membranes were incubated in phosphate-buffered saline-Tween containing 5% (w/v) skimmed milk powder, and were probed with rabbit anti-DNP as primary antibody (Molecular Probes Inc; 1:16000 dilution) and peroxidase-linked goat-anti rabbit IgG as secondary antibody (Sigma; 1:16000 dilution). Carbonylated proteins were immunodetected with a chemiluminescent peroxidase substrate, West femtoM (Pierce) [26] and visualized using a Fuji LAS1000 image analyzer with pre-cooled camera. Chemiluminescence was quantified using Fuji image gauge software.

2.4. Identification of proteins

For preliminary determination of proteins after 2D resolution, images from the SYPRO Ruby[®] stained gels were compared to the 2D yeast proteome database (<http://www.ibgc.u-bordeaux2.fr/YPM/>) and the Swiss-2D-PAGE database (<http://www.expasy.org/images/swiss-2dpage/publi/yeast-high.gif>) [27]. The identity of specific proteins within the protein array that were shown to be susceptible to carbonylation was confirmed by sequence analysis. Proteins of interest were excised from 2D gels that had been stained with 0.05 (w/v) Coomassie blue in 0.5% (v/v) acetic acid, 20% (v/v) methanol and subsequently destained with 30% (v/v) methanol. The excised proteins were digested overnight with trypsin (11 ng μl⁻¹), and the masses of the resulting peptides analyzed by matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry (Voyager DE Pro; Applied Biosystems).

Identification of each sequence was based on sequences available in the SWISS-PROT protein database [20].

3. Results

3.1. Copper exposure causes protein oxidation in cells

A Cu(NO₃)₂ concentration of 8 mM was selected for copper-treatment experiments. At this concentration the mean cell doubling time was increased by ~10% compared to non-Cu-treated controls, but there was no discernible loss of cell viability (colony-forming ability; data not shown), indicating that protein extracts were representative of all the copper-treated cells in cultures. A previous study indicated that protein oxidation in response to stress typically peaks within 15 to 45 min [28]. Consequently, our analyses focused primarily on the period 0–60 min after copper treatment. Immunochemiluminescent labeling for detection of oxidized/carbonylated proteins revealed that copper caused rapid, but transient oxidation of total soluble proteins with isoelectric points in the range of 3–10 (the range that was tested) (Fig. 1). Total carbonyl levels in these cellular proteins were unaltered after 5 min of copper treatment, but were increased by approximately eight-fold after 15 min. There was a subsequent decline in carbonylated proteins until around 60 min, when the levels of oxidized proteins were again similar to those of untreated cells. There were no significant changes in total-protein oxidation in parallel, untreated control flasks (data not shown).

3.2. Preferential copper-dependent oxidation of enzymes of the glycolytic pathway and of heat shock proteins (HSPs)

To gain insight into the oxidation of individual proteins at various times during copper treatment, cellular proteins from the relevant extracts were separated in a two-dimensional array, according to their *pI* and mass. Fig. 2A depicts a typical 2D profile of proteins from exponential phase yeast cells incubated without copper. The arrangement and relative abundances of proteins displayed corresponds well with standard yeast 2D protein profiles produced in other laboratories (see

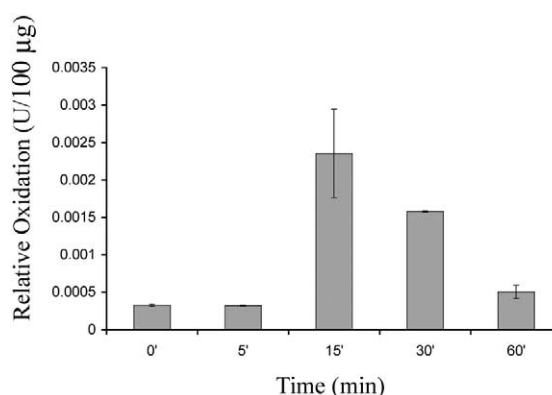


Fig. 1. Total-protein oxidation during copper exposure. Protein extracts were prepared from cells at intervals during treatment with 8 mM Cu(NO₃)₂. 2D Western blots were probed with anti-DNP antibodies and visualized with a chemiluminescent substrate to identify carbonyl groups. Oxidation was quantified using Fuji image gauge software. Total oxidation was calculated according to the sum of the chemiluminescence of all proteins in the entire blot (after background correction), and by normalizing this value against the total protein loaded onto each gel. Values reflect results from two or more 2D gel analyses, and are depicted as such in the figure, along with their standard deviation from the mean.

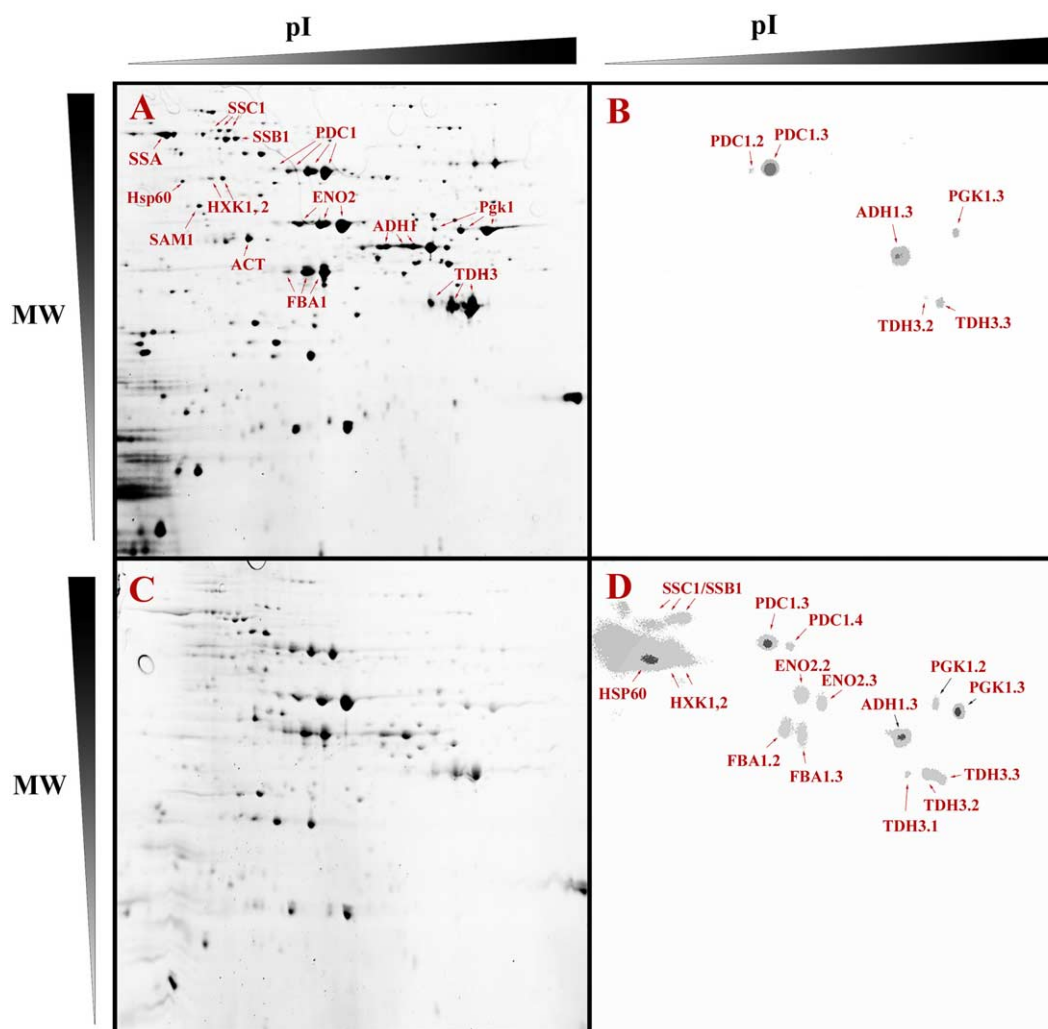


Fig. 2. 2D profiles of protein abundance and oxidation during copper exposure. Protein extracts were prepared from cells just prior to Cu exposure (panels A and B) or after 30 min treatment with 8 mM $\text{Cu}(\text{NO}_3)_2$ (panels C and D). A,C: Two-dimensional arrays of cellular proteins stained with SYPRO-ruby. B,D: Western blots of arrayed proteins probed with anti-DNP antibodies to identify oxidized/carbonylated proteins. Gels shown are typical of the results obtained from various two-dimensional protein separations prepared from cell extracts at different times after cells were exposed to copper. The relative positions of proteins that were shown to be susceptible to oxidation are referenced in panels A, B and D by their standard abbreviations.

<http://www.expasy.org/images/swis-2dpage/publi/yeast-high.gif> and <http://www.ibgc.u-bordeaux2.fr/YPM/>), in which proteins have been systematically identified by mass spectrometry [29]. Neither the expressed-protein profiles nor the corresponding profiles of carbonylated proteins varied significantly during the 60 min time course with non-Cu-treated control cells (data not shown). While the expressed-protein profile for cells that had been exposed to 8 mM copper for 30 min (Fig. 2C) was similar overall to that of non-exposed cells (Fig. 2A), enhanced copper-dependent protein oxidation was evident from the increased intensity and numbers of carbonylated proteins in immunoblots derived from extracts of copper treated cells (Fig. 2B,D).

Proteins of interest that were detected in immunoblots over the time course of copper exposure were characterized further. In all cases, the distributions of proteins in gels and blots were in excellent agreement with each other, and with the available 2D gel databases (see Section 2). Nevertheless, the identity of each protein of interest was validated by MALDI-mass spectrometry (Table 1). Strikingly, of the 10 detectable protein

types that were oxidized at some stage during copper treatment, eight were found to be involved either directly in glycolysis or in subsequent catabolic reactions (Table 1; Fig. 3). The marked susceptibility of HSPs to oxidation was also apparent (Fig. 2D; Table 1). Indeed the extent of HSP oxidation, after 15 min or more precluded any consistent resolution of the oxidation levels for any of the individual proteins (exemplified in Fig. 2D). Consequently these proteins were treated as a family of proteins, and their collective abundance and oxidation profiles are reported as such (Table 1). The proximity of these HSPs on the 2D Western blots to hexokinase 1 (Hxk1), hexokinase 2 (Hxk2) and *S*-adenosylmethionine synthetase 1 (Sam1) also prevented any determination of the oxidized levels of these enzymes after 15–30 min; effectively nullifying any temporal evaluation of their susceptibility to oxidation.

3.3. Differing susceptibilities of individual protein targets over time to copper-dependent oxidation

In order to characterize each of the proteins that was most

Table 1

A list of all the proteins that exhibit some degree of carbonylation within 60 min of the cells being exposed to 8 mM copper

	Std. name	Protein name	MW (kDa)	~pI
1	Act1	Actin	41.7	5.19
2	Adh1	Alcohol dehydrogenase 1	36.8	6.06
3	Eno2	Enolase 2	46.8	5.56
4	Fba1	Fructosebiphosphate aldolase	39.5	5.39
5	Tdh3	Glyceraldehyde-3-phosphate dehydrogenase 3	35.6	6.50
6	Hxk1 and 2 ^a	Hexokinase 1 and 2	53.6	5.07
7	HSPs ^a (Hsp60, Ssa1, Ssa2, Ssb1, Ssc1)	Heat shock proteins ^a		
8	Pdc1	Pyruvate decarboxylase 1	61.4	5.62
9	Pgk1	Phosphoglycerate kinase 1	61.2	5.89
10	Sam1 ^a	S-adenosylmethionine synthetase 1	41.7	4.79

^aDenotes a protein or series of proteins which exhibited varying degrees of carbonylation, but the degree of oxidation was difficult to denote/differentiate throughout the time course of the experiment.

susceptible to copper-dependent oxidation at each time point, data for individual proteins from each gel and Western blot were normalized against the total-soluble-protein concentration, quantified on SYPROrubySM-stained gels at each time point. In so doing, it was apparent that different proteins exhibited marked differences in copper-dependent carbonylation, both in degree and in time dependence (Fig. 3). This diversity of oxidative effects on individual proteins is reflected in the fact that only Adh1 and Tdh3 exhibited a pattern of

oxidation that closely resembled the averaged trend seen for the total protein (Figs. 1 and 3). Several other proteins, however, also showed significant increases in oxidation after the cells had been exposed to copper, namely: Fba1, Eno2, Pdc1, Pgk1 and Act1. Maximal oxidation of these proteins was evident after 30 min (Fig. 3). Curiously, the abundance of individual proteins (relative to total loaded protein) often exhibited an increase after 15 min, before returning to pre-copper levels after 60 min. The effect was most striking for Eno2 and

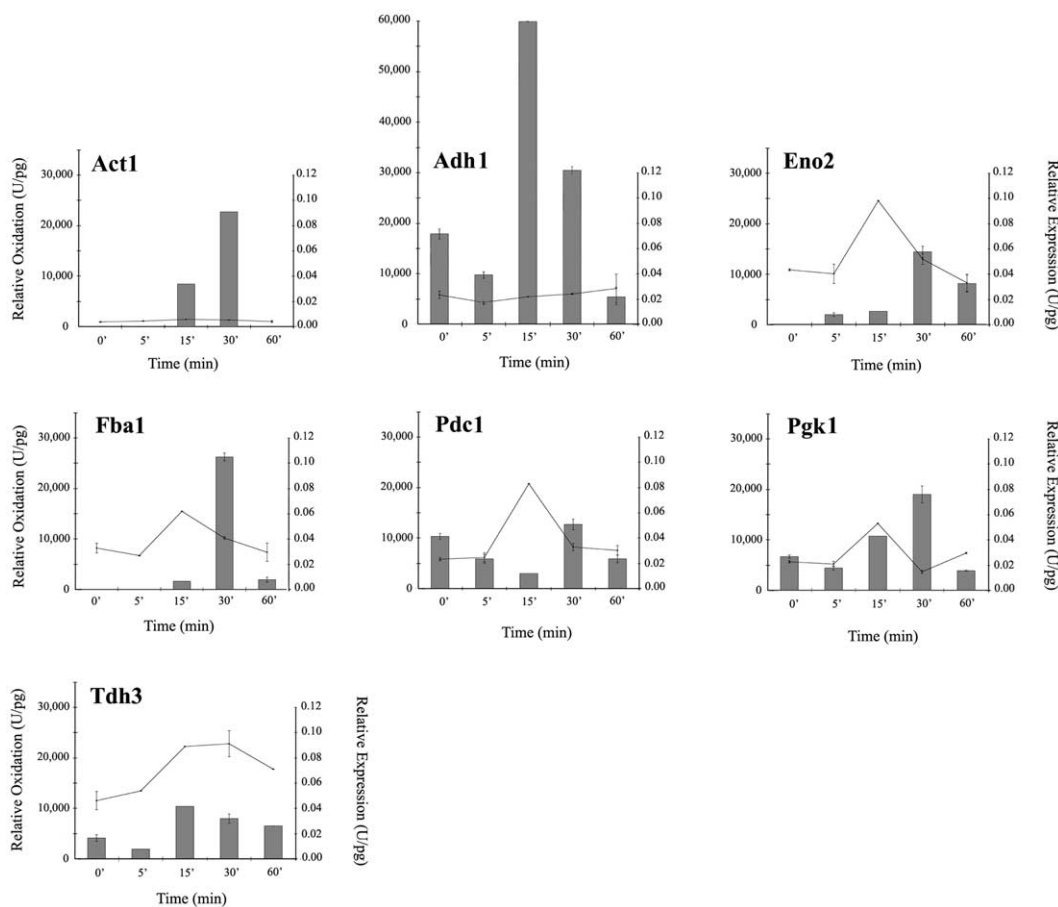


Fig. 3. Oxidation levels and abundances of individual proteins during copper exposure. The oxidation levels (histograms) and protein abundances (lines) of each targeted protein were quantified in extracts prepared from cells at intervals during exposure to 8 mM $\text{Cu}(\text{NO}_3)_2$. Owing to the inherent limits of 2D Western analysis, data for the HSPs, Hxk and Sam1 are not presented here. The oxidation levels shown for individual proteins are normalized against their relative abundance at the corresponding time points, and subsequently expressed as a function of total protein. Values reflect results from two or more independent gel separations (whenever possible) and are depicted along with their standard deviation.

Pdc1, each of which exhibited a > 2.5-fold change in relative abundance during this time (Fig. 3). In contrast, levels of Act1 and Adh1 (Fig. 3), Hxk1, Hxk2, Sam1 and the HSPs (data not shown) were relatively constant throughout.

3.4. Differing susceptibilities of individual isoforms of protein targets to copper-dependent oxidation

With the exception of Hxk1, all the glycolysis-related enzymes that were targeted for carbonylation during copper stress were present in the cell extracts as three or more distinct isoforms, whose profile could be discerned on 2D gels (Fig. 2).

The relative abundance of the individual isoforms of each protein and their apparent susceptibilities to copper-dependent oxidative damage differed markedly. Thus, for example, the peak level of carbonylation was clearly greater for Adh1.1 and Pgc1.1 than for the other detected isoforms of these two proteins (Fig. 4). Moreover, with the possible exception of Adh1, the most-abundant isoform of each enzyme consistently appeared to be the one that was targeted least by copper-dependent oxidation. By way of example, mean levels of Eno2.3 were more than two-fold higher than those of either Eno2.1 or Eno2.2 during the time course examined, whereas peak car-

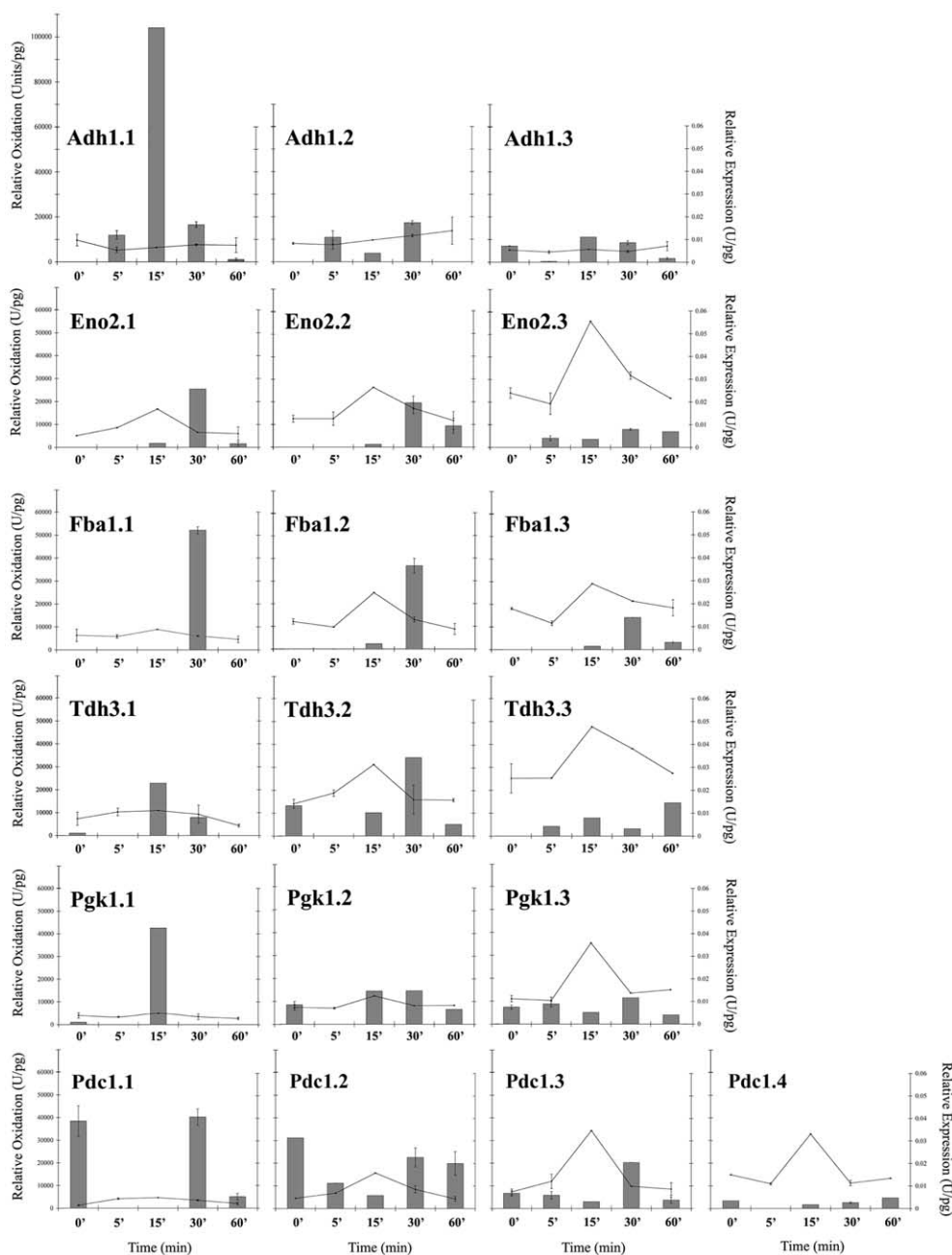


Fig. 4. Oxidation levels and abundances of individual isoforms of targeted proteins during copper exposure. The oxidation levels (histograms) and abundances (lines) of each targeted protein were quantified in extracts prepared from cells at different intervals during exposure to 8 mM $\text{Cu}(\text{NO}_3)_2$. The oxidation levels shown for individual isoforms are normalized against their relative abundance at the corresponding time points, and subsequently expressed as a function of total protein. Values shown reflect results from two or more independent gel separations (whenever possible) and are depicted along with their standard deviation.

bonylation of Eno2.3 was more than two-fold lower than in the other isoforms.

4. Discussion

In this study we demonstrate that exposing *S. cerevisiae* to sublethal levels of copper inflicts oxidative damage on a number of its cytoplasmic proteins. The level of oxidation peaked within 15 to 30 min after copper addition, establishing a temporal component to copper-induced protein oxidation. The majority of proteins that were oxidized over the course of the investigation also demonstrated an increase in their relative abundance after 15 min of exposure. The subsequent decline in abundance after 15 min was coincident with the maximal oxidation levels of most copper-targeted proteins. The transient peak in levels of oxidized proteins was similar to previous observations [28], and consistent with selective degradation of oxidatively damaged proteins [20]. Such a correlation, however, was not always observed in this investigation. Adh1 (the most heavily oxidized enzyme of all those identified) showed no evidence of altered levels of expression or degradation throughout the investigation, indicating that protein degradation need not be a necessary consequence of excessive or directed oxidation.

This study also demonstrated that, in a similar manner to peroxide-induced oxidative stress, copper treatment resulted in the specific oxidation/carbonylation of a discrete subset of predominantly catabolic enzymes. While the targeted gene products also included proteins such as actin and several HSPs, the majority of proteins which demonstrated a clear and specific susceptibility to oxidation during copper exposure were enzymes involved either directly in glycolysis, or in the fermentation of the glycolytic product, pyruvate. A similar targeting specificity has recently been observed during peroxide-induced oxidative stress in *S. cerevisiae* [19,22], wherein, low levels of hydrogen peroxide elicited the preferential damage and inhibition of a more limited set of glycolytic enzymes. These similarities add to the previously documented overlaps in other aspects of the copper- and oxidative-stress responses of yeast [9–12] and lend further support to the hypothesis that oxidative mechanisms underpin copper toxicity [16].

Among the catabolic enzymes shown here to be susceptible to copper-dependent oxidation, all (except Hxk, Pgk1 and Pdc1) have also been shown to be targets for protein *S*-thiolation – an activity that serves to protect proteins from irreversible oxidative damage [22]. This association between susceptibility to oxidation and preferential *S*-thiolation persists even among isoenzymes of a single protein species. Grant et al. [30] demonstrated that of the three different isoenzymes of glyceraldehyde 3-P-dehydrogenase expressed in *S. cerevisiae* (Tdh1, Tdh2 and Tdh3), only Tdh3 was specifically modified by *S*-thiolation. Similarly in the present study, copper-induced oxidation was detectable only in Tdh3 and not in either Tdh1 or Tdh2, despite the presence of the latter two isoenzymes in the protein array. While it may appear logical that oxidation-susceptible proteins should be the ones that merit specific protection mechanisms, it is noteworthy that the interplay of such opposing actions (specifically, oxidative damage versus scaleable protection against oxidative damage) provides a rather classic regulatory mechanism for a finely controlled cellular response to oxidative stress. This regulatory interplay is all the more plausible, given the collective and individual

dynamics of the oxidation profile of the different proteins within the first 60 min after copper exposure (Figs. 1 and 4), and the time (approximately 1 h) after which oxidative damage and *S*-thiolation of these proteins was demonstrated in the hydrogen peroxide stress-induced cells [20,22].

The oxidation of Adh1 affords a mechanism by which fermentation of glucose (the preferred metabolism of yeast grown on glucose [31]) can be rapidly influenced. This, along with the apparent, directed oxidation of the other glycolytic pathway enzymes, is consistent with a cellular metabolic response to other forms of oxidative stress that has been promoted by a number of groups [20,22,32,33]. This response involves a transient metabolic re-shuffling of glucose equivalents through the pentose phosphate pathway (resulting from the targeted inactivation of specific glycolytic enzymes). It has been proposed that this metabolic shift could provide the necessary reducing power (NADPH₂) for antioxidant enzymes such as those of the glutaredoxin systems [19,34,35]. In addition, the directed oxidation of glycolytic enzymes may temporarily alleviate the repressive effects of glucose metabolism on cellular antioxidant defense mechanisms, such as the transcriptional induction of catalase, superoxide dismutase and/or glutathione peroxidases [36,37]. Thus, even in cells using glucose as the sole carbon source, a transient cessation of glucose metabolism might facilitate cellular defense against oxidative stress. While the oxidative data is incomplete, a similar argument can be made for the preferential oxidation of the only non-glycolytically related enzyme that was shown to be oxidized in this analysis, Sam1 (Table 1). Given the importance of glutathione as an antioxidant, and its presumed role in metal resistance in yeast [38–40], a transient inactivation of Sam1 by oxidation could serve to divert the sulfur amino acid biosynthetic pathway away from the formation of *S*-adenosyl homocysteine and toward the production of glutathione. Whatever the mechanism(s), directed oxidation of the glycolytic and fermentative enzymes as well as Sam1 seems likely to be beneficial to cells in countering copper-induced oxidative stress.

The precision that is apparent in the targeting of specific metabolic enzymes during copper exposure is even more clearly demonstrated by the differing susceptibilities of individual isoforms of these enzymes to oxidation. Similar isoform-specific responses have been reported previously. For example, apolipoprotein E in oxidatively stressed human cells exhibits isoform-specific free-radical-scavenging activity [41, 42]. In regard to copper-induced oxidation, it is of particular interest that the most-abundant isoform of each enzyme was the least affected by copper-dependent oxidation (Fig. 4). This is even more remarkable when one considers that (with the notable exception of Adh1) all the targeted catabolic enzymes experience a significant increase in their relative abundance within 15 min after exposure to copper (Fig. 3). While the precise association of protein oxidation and its effect upon the function of each enzyme still need to be determined, the multimeric nature of the targeted enzymes may provide a clue as to how oxidation of one isoform could have an impact upon the stability of another, and thus the function of the intact enzyme. Indeed, were the *transient* reduction of glycolytic activity to be the goal (resulting in a transitory re-routing of carbon units through the pentose phosphate pathway), then perhaps oxidation of the least-abundant isoform would provide an ideal means to disrupt enzyme function, while still

retaining a significant proportion of unmodified subunits to allow for the subsequent, rapid restoration of optimal glycolytic metabolism. Further work is required to substantiate this additional, possible layer of targeted response to copper stress.

Whatever the underlying mechanisms or consequences of protein oxidation, the results shown in this study clearly demonstrate that exposure of *S. cerevisiae* to sublethal levels of copper induces an oxidative stress that is similar in scope and specificity to that induced by hydrogen peroxide and other ROS. Furthermore, the results also indicate that this copper-induced oxidative stress occurs within the first hour of exposure, and is a dynamic process that specifically targets key enzymes involved in the fermentative catabolism of glucose.

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